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Schistosoma haematobium: Identification of new estrogenic molecules with estradiol antagonistic activity and ability to inactivate estrogen receptor in mammalian cells

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ABSTRACT

We have previously identified the expression of an estradiol (E2)-related molecule by *Schistosoma haematobium* total antigen (Sh). We now show that this molecule has an antagonistic effect of estradiol in vitro. Our results are consistent with the existence of an estrogenic molecule that antagonizes the activity of estradiol. We found evidence for this molecule as we identified and characterized by mass spectrometry new estrogenic molecules previously unknown, present in schistosome worm extracts and sera of *Schistosoma*-infected individuals. We also show that Sh is able to interact in vitro with estrogen receptor (ER), explaining how host endocrine system can favor the establishment of schistosomes. These findings highlight the exploitation of the host endocrine system by schistosomes and represent an additional regulatory component of schistosome development that defines a novel paradigm enabling host–parasite interactions. The identification of these molecules opens new ways for the development of alternative drugs to treat schistosomiasis.

1. Introduction

Despite affecting 200 million people, schistosomiasis remains a neglected disease. There are two main reasons for this: the availability of an effective and cheap drug, praziquantel, has contributed to the wrong perception that chemotherapy would represent the final solution for the control of schistosomiasis, and the disability adjusted life years as a measure of the impact of disease has been under evaluated because of rebound morbidity following chemotherapeutic intervention (Capron et al., 2005). Prevalence is thought to be rising mainly due to increasing travelers from the USA and Europe to these endemic regions for business or leisure. Wars are also known to increase the impact of schistosomiasis as demonstrated by a case recently published by our group (Vieira et al., 2007).

The mechanisms by which host hormones act on parasites have recently been investigated, and some parasite molecules that are involved in transregulation have been identified and characterized. In the case of schistosomes, hormonal signals from the host seem to have a major influence on larval homing, survival, growth and sexual maturation (Mendonça et al., 2000; Escobedo et al., 2005). In the case of schistosomes, some hormonal signals from the host seem to have a major influence on larval homing, survival, growth, and sexual maturation. Moreover, this is a 2-way dialogue and certain helminth parasites can have a particularly marked effect on the fecundity of the host (Mendonça et al., 2000).

Nuclear hormone receptors comprise a large superfamily of transcription factors whose activity is under hormonal control. These receptors are characterized by a central DNA-binding domain, which interacts with specific hormone response elements located near the target gene promoter, and by two distinct activation function (AF) domains that contribute to the transcriptional activity of these receptors (Delage-Mourroux et al., 2000).

The estrogen receptor (ER), a member of the steroid receptor family, mediates the stimulatory effects of estrogens and the

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inhibitory effects of antiestrogens in estrogen target cells (Martini et al., 2000). Estrogen receptor (ER), mediates the biological effects of estrogens in a variety of target tissues (Delage-Mourroux et al., 2000) and ER-regulated genes are involved in many biological processes, including cell growth and differentiation, morphogenesis, and programmed cell death (Martini et al., 2000). The bound estrogen receptor stimulates transcription via interaction with the estrogen response element (ERE) in the nucleus (Catherino and Jordan, 1995).

We recently reported the expression of an estradiol-like molecule by a trematode parasite *Schistosoma haematobium* (Botelho et al., 2009). To better understand the mechanism by which this molecule works, we transfected CHO cells with an expression plasmid containing an ERE linked to a luciferase and treated the cells with *S. haematobium* total antigen (Sh). In this way, as we show in this paper, we identified interaction of Sh with the ER. Intriguingly, we found that the estradiol-like molecule is an antagonist of estradiol, repressing the transcriptional activity of the ER. To better characterize this molecule we recurred the technique of mass spectrometry and developed a new method for the detection of estrogenic molecules in biologic samples. In this way, as we show in this paper, we identified new estrogenic molecules in *S. haematobium* total antigen as well as in the serum of infected individuals with this parasitic disease that seem to be specific of this parasite.

Elucidating the role of estrogens signaling pathway during *S. haematobium* infection will determine the use of new drug targets to treat this disease.

2. Materials and methods

2.1. Animals

Eight-week-old female golden hamsters (LVG/SYR) were provided by Charles River (Barcelona, Spain). Animals spent one week being acclimated under routine laboratory conditions before starting the experiments. They did not receive any treatment prior to the study. Hamsters were kept in separated cages and fed standard balanced food and water ad libitum. All the animals were raised and maintained at the National Institute of Health (Porto, Portugal) in rooms with controlled temperature (22 ± 2 °C) and humidity (55% ± 10%) and continuous air renovation. Animals were housed in a 12 h light/12 h dark cycle (8 am to 8 pm). All animal experiments were performed in accordance with the National (DL 129/92; DL 197/96; P 1131/97) and European Convention for the Protection of Animals used for Experimental and Other Scientific Purposes and related European Legislation (OJ L 222, 24.8.1999).

2.2. Parasites

Schistosoma haematobium (Angolan strain) life cycle was maintained through successive passages in laboratory-raised *Bulinus truncatus* as invertebrate hosts and golden hamsters as vertebrate hosts. Cercariae of *S. haematobium* were obtained from infected snails by the use of artificial light.

2.3. Experimental infections

Urine was collected from *S. haematobium*-infected individuals. The individuals were living in Angola, an endemic area for schistosomiasis. Following instruction in midstream urine collection, urine samples were then collected from each individual. *S. haematobium* infection was detected by microscopically observation of the eggs in the sediment of centrifuged urine. Informed consent from patients was obtained. The eggs were hatched and with the resulting miracidia, snails from susceptible species were infected. Golden hamsters were experimentally infected with 100 cercariae by tail immersion. The control animals consisted of littermates. The cercariae were obtained by shedding of snails infected with miracidia.

2.4. Schistosoma haematobium total antigen production

Schistosoma haematobium adult worms were collected by perfusion of the hepatic portal system of golden hamsters at seven weeks after infection with 100 cercariae. The worms were suspended in PBS and then sonicated. The protein extract was then ultracentrifuged (20,000 RPM) and the protein concentration was estimated using a micro BCA protein assay reagent kit.

2.5. Sera

Sera from *S. haematobium*-infected individuals were obtained from CIBP (INSA-Porto). All sera were selected on the basis of positive urine egg counts for *S. haematobium*.

2.6. Chemicals and materials

CHO cells were cultured in CHO medium (Sigma Aldrich, Portugal) and MCF-7 cells were cultured in RPMI 1640 medium phenol red free (Sigma Aldrich, Portugal), with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin, purchased in Invitrogen Life Technologies (Paisley, Scotland, UK). Estradiol (E2) was obtained from Sigma Aldrich (Portugal) and the antiestrogen ICI 182,780 (ICI) was kindly provided by AstraZeneca (Portugal), both chemicals dissolved in 100% ethanol and added to cell culture medium.

2.7. Plasmids

Plasmids were used to transform DH5α *Escherichia coli*. Individual colonies were isolated and plasmid miniprep was done using the Promega Miniprep Kit. The desired plasmid was amplified and isolated using the Qiagen maxiprep kit. The estrogen-responsive reporter plasmid, pERE-Luc, was kindly provided by Ming Tsai and Bert O'Malley (Baylor College of Medicine, Houston, TX) (Nawaz et al., 1999).

2.8. Cell culture

CHO cells and MCF-7 cells were maintained at 37 °C in a 5% CO_2 humidified atmosphere. Cells were passaged every 5 days. Before treatments cells were serum-starved for 16 h. The cells were treated with Sh antigen for 24 h in serum-free conditions.

2.9. Estradiol assay

CHO cells were cultured in 0.3 ml CHO media in 24-well flat-bottomed plates. Cultures were seeded at 5×10^4 cells/well and allowed to attach overnight. Cells were then treated with increasing concentrations of Sh for 24, 48 and 72 h. Estradiol in the supernatant of CHO cells was determined using electrochemiluminescence (ECLIA) with the Immulite 2000 Analyser (DPC) and ELISA (Calbiotech) according to the manufacturer's instructions.

2.10. Lactoferrin assay

MCF-7 cells were cultured in 0.3 ml RPMI medium in 24-well flat-bottomed plates. Cultures were seeded at 5×10^4 cells/well and allowed to attach overnight. Cells were then treated with 50 μg/ml of Sh for 24 h. Lactoferrin in the supernatant of MCF-7 cells was determined using ELISA with an antibody specific for

lactoferrin (Hycult, Netherlands) according to the manufacturer's instructions.

2.11. Isolation of RNA

At defined time intervals MCF-7 cultured cells were harvested in Tripure reagent (Roche, Portugal). Total RNA was isolated from the cell material according to the manufacture instructions. RNA recovery and quality were checked by measuring the optical density ratio (260/280 nm) and its quantification was determined also by optical density (Nanodrop).

2.12. Real-Time PCR

Real-Time PCR was performed in MCF-7 cultures. For this purpose cDNA was obtained using the Promega cDNA kit. For each sample, 1.0 μg of RNA was reverse transcribed in a reaction volume of 20 μl in the presence of 10 mM deoxynucleotide triphosphate and 2 μl RT-PCR enzyme mix. Taqman GeneExpression assays (Applied biosystems) were used for gene-specific primers and probes for ER alpha (*ESR1*, assay ID Hs 01046817_m1, PE Applied Biosystems, USA) and ER beta (*ESR2*, assay ID Hs 00230957_m1, PE Applied Biosystems, USA). Both target gene transcripts were normalized to the reference gene GAPDH mRNA (assay ID Hs 99999905_m1, PE Applied Biosystems, USA) content and to the control sample. Quantifications were done in triplicate in a 7500 Real-Time PCR System (PE Applied Biosystems, USA).

2.13. Transient transfection

CHO cells were plated for transfection in 24-well plate and incubated for 24 h at 37 °C with 5% CO_2 the day before transfection. The cells were transfected with 0.75 μg ERE-Luc plasmid per well and 0.3 μg pCMV-β (which constitutively produces β-galactosidase) for normalization. Cells were transfected by using Lipofectamine (Invitrogen, Carlsbad, California, USA) according to the manufacturer's recommended guidelines. Cells were washed and fed with CHO medium and subsequently treated with 50 μg/ml of Sh, 10^{-9} E2 and 10^{-9} M ICI. Concentrations for E2 and ICI used were based on previous studies (Soares et al., 2003). As a control, cells were treated with vehicle alone (ethanol). After 24 h, cells were harvested and cell extract were prepared for luciferase assay. Luciferase activity was determined using an enhanced luciferase assay kit (BD Biosciences, USA) in a scintillation counter (Amersham Biosciences, USA). Cell extracts were assayed for β-galactosidase activity as described previously (MacGregor and Caskey, 1989), and the luciferase units were normalized to β-galactosidase activity from the cotransfected pCMV-β internal reference plasmid.

2.14. Mass spectrometry analysis

LC–MS analysis were carried out using a Thermo Finnigan Surveyor LCQ DECA XP MAX quadrupole ion trap mass spectrometer, utilizing electrospray ionization, coupled to a LC of Thermo Finnigan. UV detection was achieved at 280 nm and the column was a Merck Purospher STAR RP-18e 125 cm × 4.6 (3 μm), equipped with a Merck Lichrocart pre-column (Merck, Germany).

The mobile phases consisted of water/acetonitrile/acetic acid mixtures, 95:5:0.5 for A and 45:55:0.5 for B. The following gradient was 0–5 min, 100% A; 5–10 min, linear gradient from 100% to 80% A; 10–15 min, 80% A; 15–50 min, linear gradient from 80% to 40% A; 50–65 min, 40% A; 65–75 min, linear gradient from 40% A to 100% B. The flow rate was 0.5 mL/min and for MS^2 experiments, the collision energy was optimized at 30%.

2.15. Statistical analysis

Data were expressed as mean ± SD. *t*-Test was used to assess the statistical significance of differences. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Schistosoma haematobium total antigen decreases estradiol production of CHO cells in vitro

Since we discovered that Sh expresses a molecule analogue to estradiol (Botelho et al., 2009), we developed an assay to confirm the estradiol-like effect on estrogen responsive cells. Here we evaluate the production of estradiol by these cells, since estradiol production is a target of estradiol itself. For this purpose, CHO cells were seeded on 96 well plates, starved ON, treated with increasing concentrations of *S. haematobium* total antigen (Sh) for 24 h, cultured for 24, 48 and 72 h and their supernatants analyzed by ECLIA. Surprisingly, the curve shows that cells treated with Sh at a concentration of 50 μg/ml or higher decreased significantly estradiol production in comparison to control cells (Fig. 1).

3.2. Schistosoma haematobium total antigen is an antagonist of estradiol

To further elucidate the previous results, we analyzed the effects of estradiol (E2), ICI 182,780 (ICI) and Sh on estradiol production in CHO cells. Serum-starved cells in six-well plates were treated with 10^{-9} M E2, 10^{-9} M ICI or 50 μg/ml of Sh for 24 h. Supernatants were analyzed by ELISA. We observed, as expected, that ICI reverts estrogen release to the supernatant (E2 + ICI compared to E2 bars). Surprisingly, Sh decreases significantly estradiol level in supernatant to a higher extent than ICI. These findings demonstrate an antagonist effect of estradiol. Remarkably, co-culturing Sh with ICI reverts the decrease of estradiol induced by Sh, demonstrating that this effect is probably mediated by estrogen receptor (Fig. 2).

3.3. Schistosoma haematobium total antigen reduces the production of lactoferrin in the supernatant of MCF-7 cells

Lactoferrin is a target gene of estrogen receptor and can be a useful marker of estrogen action on estrogen responsive cells like MCF-7. Therefore MCF-7 cells were seeded on 96 well plates, starved ON, treated with 50 μg/ml of Sh for 24 h, cultured for 24 and 48 h, and the supernatants analyzed by ELISA for lactoferrin (Fig. 3). Bars show that Sh-treated cells produced less lactoferrin than control cells. In addition, incubation with ICI or Sh + ICI also led to identical decrease in lactoferin secretion ($^*p < 0.05$ vs

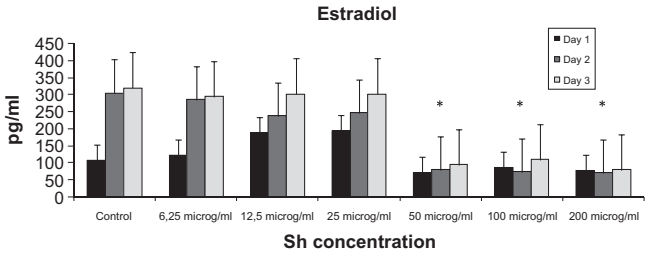


Fig. 1. Estradiol production in culture of *S. haematobium* total antigen treated CHO cells. The experiments were done in triplicate ($^*p < 0.01$; 50, 100 and 200 μg/ml vs. control) for days 1, 2 and 3. We used the concentration of 50 μg/ml of Sh for the subsequent assays.

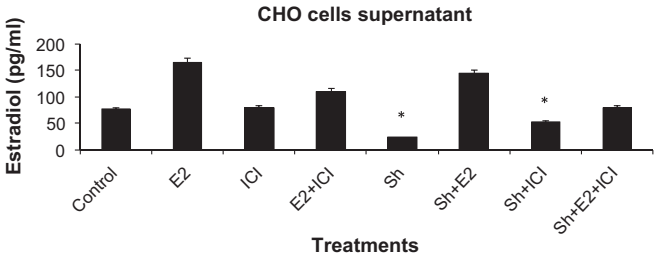


Fig. 2. Estradiol production in supernatant of CHO cells. Cells were treated with 10–9 M of E2, 10–9 M of ICI and 50 mg/ml of Sh. Data represent means ± SEM from three independent experiments (*$p < 0.05$ Sh vs. control).

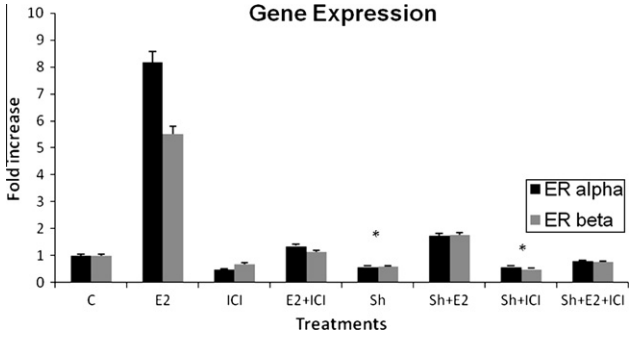


Fig. 4. Real-Time PCR for ER alpha and ER beta in MCF-7 cells. Bars correspond to fold increase of ER alpha and ER beta gene expression after normalization to endogenous control gene GAPDH and control sample. Results are representative of three independent experiments. Statistically significant differences in ER alpha and ER beta gene expression were found between Sh-treated and vehicle-treated MCF-7 cells (*$p < 0.05$ vs. control).

Controls).These results suggest that Sh treatment represses estrogen receptor activity.

3.4. Schistosoma haematobium total antigen down-regulates ER alpha and ER beta

To confirm the involvement of estrogen receptor, expression of ER alpha and ER beta were analyzed in MCF-7 cells after estradiol, ICI and Sh treatment. Real-Time PCR analysis for ER alpha, ER beta, and endogenous control gene GADPH were carried out by using RNA derived from either vehicle-treated (control), estradiol-treated (E2), ICI-treated (ICI) or Sh-treated in MCF-7 cell cultures. Incubation with estrogen clearly increased both ERalpha and ER beta expression (Fig. 4). Furthermore, both ER isoforms were down-regulated by Sh treatment in this cell culture, either alone or in the presence of ICI in comparison to control (Fig. 4). Interestingly, Sh incubation extensively reduced ER alpha and ER beta expression, even in the presence of estrogen (*$p < 0.05$ Sh + E2 vs. E2).

3.5. Schistosoma haematobium repression of ER transcriptional activity

Nuclear hormone receptors regulate gene expression by interacting directly with DNA response elements. To confirm Sh-mediated down-regulation of ER we carried out transfection of CHO cells with a reporter gene construct containing an estrogen response element (ERE). As shown in Fig. 5, Sh effectively reduced the transcriptional activity of the ER. When cells are treated simultaneously with Sh and ICI there is a synergistic effect in the reduction of luciferase activity. Sh was also able to significantly reduce ER transcriptional activity in the presence of E2 (Fig. 5) as revealed by comparing Sh + E2 with E2 results.

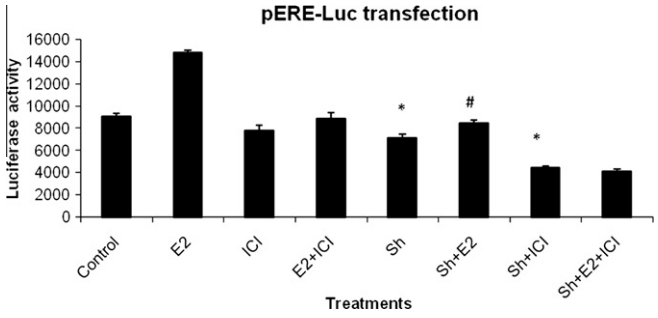


Fig. 5. Role of estradiol on activation of estrogen responsive element (ERE) was assessed by luciferase assay. CHO cells were transfected with a pGL3 reporter construct containing an ERE linked to luciferase, and incubated with 10^{-9} M E2, 10^{-9} M ICI or 50 μg/ml Sh afterwards. Reporter gene transactivation was evaluated 2 days after transfection. Luciferase activity induction was measured. Incubation with Sh or in the presence of ICI resulted in a statistically significant decrease in luciferase activity compared to control (*$p < 0.05$ Sh vs. control; Sh + ICI vs. control). Co-incubation of Sh together with E2 further reduced E2 effects on ER transcription activity (#). Bars represent mean values of three distinct assays. Error bars represent SD. Experiments were performed in triplicate.

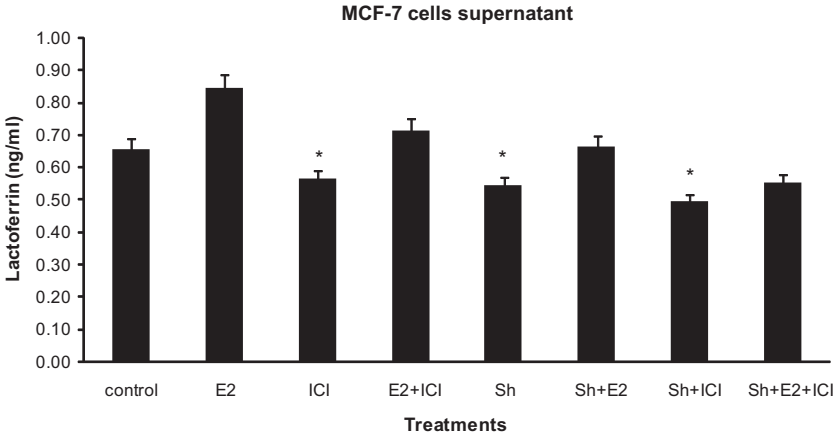


Fig. 3. Lactoferrin production in supernatant of MCF-7 cells. Cells were treated 50 μg/ml of Sh. Data represent means ± SEM from three independent experiments (*$p < 0.05$ Sh vs. control; ICI vs. control; and Sh + ICI vs. control).

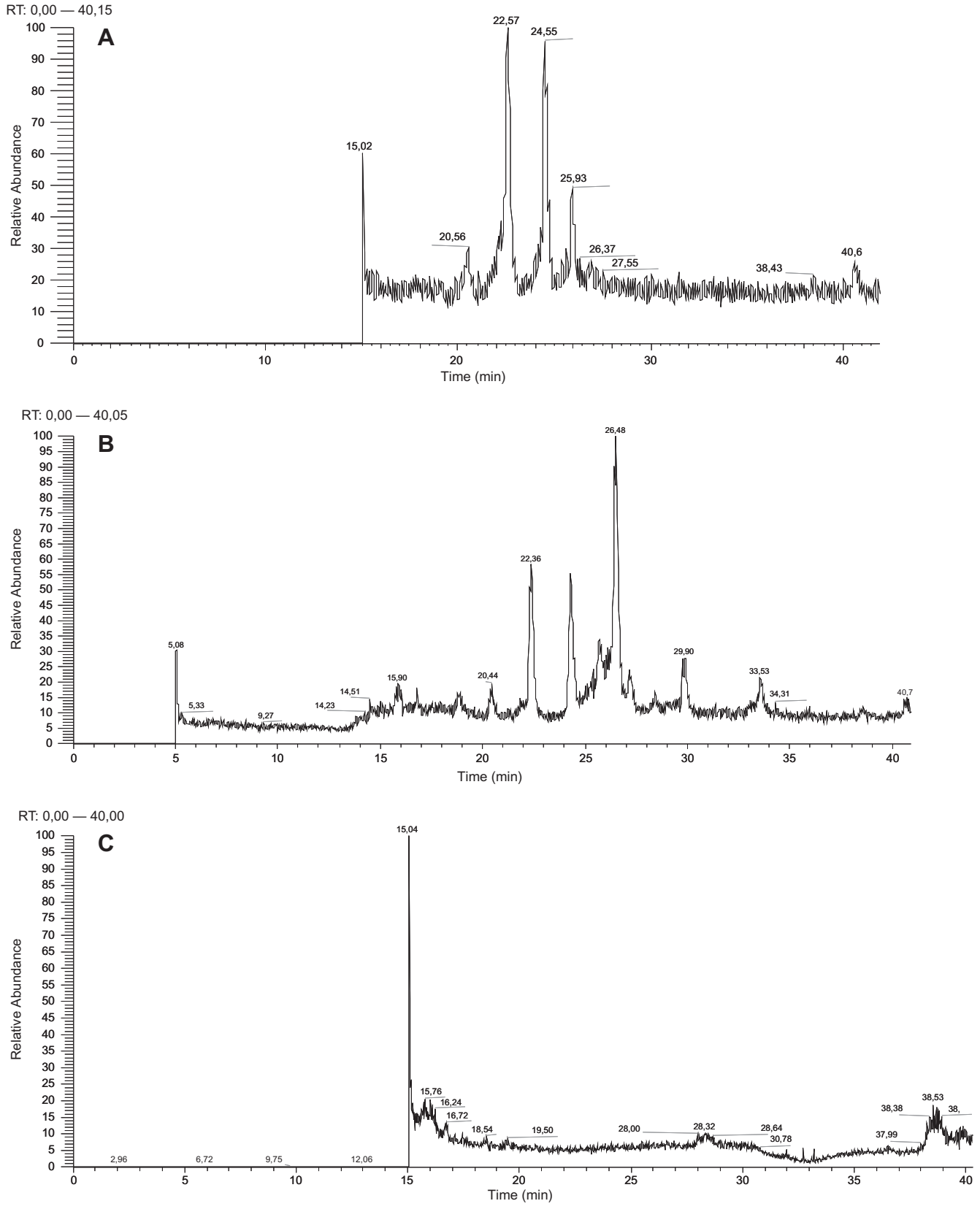


Fig. 6. LC–ESI–MS analysis of schistosoma extract (A), serum from a *Schistosoma*-infected individual (B), and serum from a healthy donor (C). MS spectra of main components in samples analyzed are given in Supporting Information.

Table 1
Retention times and *m*/*z* values for the four main components identified by LC–MS analysis of schistosoma samples.

Component	Retention time (min)	"Duplicated" *m*/*z* peaks detected on negative mode
1	20	610/624
2	22	723/737
3	24	836/850
4	26	949/963

3.6. Identification of new estrogenic molecules in S. haematobium total antigen

Liquid chromatography coupled to electrospray ionization mass spectrometry (LC–ESI–MS) was used to provide both a clear distinction between different samples and a structural identification of their main components. Fig. 6 depicts chromatograms obtained for biological samples by LC–ESI–MS. As seen, both the schistosoma extract (Fig. 6A) and the plasma from a *Schistosoma*-infected individual (Fig. 6B) present four main peaks with retention times of approximately 20, 22, 24 and 26 min. Interestingly, these four peaks were not observed for the plasma from a healthy donor (Fig. 6C). Mass spectral analysis, run in the negative mode (i.e., species detected as [M-H]-ions), of the four main components present in both schistosoma samples revealed that those were species with molecular weights considerably higher than that of estradiol (Table 1). Moreover, the corresponding *m*/*z* peaks seemed duplicated (see MS spectra on Supporting Information), as each component was invariably related to two MS peaks, always detected at *m*/*z* ([M-H]-) and *m*/*z* ([M-H]-) + 14. Species detected after MS^2 fragmentation of components **1**–**4**, as illustrated in Fig. 7 for component **2** – is the most intense in the schistosoma extract – gave further support to our previous structural assignment for the four components.

4. Discussion

The transcriptional activity of ER is now well described to be negatively regulated by *S. haematobium*. The present studies document that Sh represses the activation of ER-induced estrogen responsive element. We also attempted to identify the molecule(s) in the parasite extract responsible for this effect.

The studies presented in this work indicate that Sh expresses estrogenic molecules that interact with ER by an intriguing mechanism, by down-regulating and repressing ER transcriptional activity. Our experiments show that there is a direct interaction of Sh, with ER and that this interaction is ligand-dependent. The ability of Sh to decrease transcriptional activity by the ER was observed with a reporter gene construct containing an ERE.

In this manuscript, we present data suggesting that Sh modulates the concentration of ER transcripts in the estrogen responsive MCF-7 cells, by promoting its down-regulation in a similar way as the estrogen inhibitor ICI. The hypothesis that this estrogen antagonistic effect observed in MCF-7 cells could be due to contamination of Sh extract by host animal cells that eventually would interfere with estrogen signaling was ruled out by the procedure used for Sh isolation. Worms were obtained by perfusion of golden hamster hepatic portal system, washed in saline solution, counted and sonicated in order to obtain the antigen. In addition, Sh was frozen. Therefore, the whole procedure would completely destroy host cell contamination. We used Real-Time PCR to determine the expression of ER alpha and ER beta mRNA MCF-7 cells treated with Sh, E2 and ICI in comparison to vehicle-treated control cells. Sh clearly down-regulates ER alpha and ER beta. The estrogen receptor (ER) exists in two isoforms ER alpha and ER beta with a different distribution in the body and different functions, which are not clearly identified yet (Zimmermann and von Angerer, 2007). Physiological effects of estrogen are mediated through both ER alpha and beta. Importantly, ER alpha- and ER beta-dependent pathways regulate distinct and largely nonoverlapping sets of genes. Whereas ER alpha is essential for most of the estrogen-mediated increase in gene expression, ER beta mediates the large majority (nearly 90%) of estrogen-mediated decreases in gene expression (O'Lone et al., 2007). Additionally, it is possible that Sh-induced ER alpha and beta down-regulation serves to control physiological responses in estrogen target tissues, which ultimately serves to limit the expression of estrogen responsive genes by the parasite.

Interestingly, Sh which enhances the inhibitory effectiveness of antiestrogens (Figs. 4 and 5), also moderates the activity of estrogens because Sh is recruited to the ER even in the presence of estradiol, exerting its antagonist actions. Therefore our findings imply that Sh dampens the stimulatory response to estradiol.

Our results are consistent with the existence of an estrogenic molecule that antagonizes the activity of estradiol. We found evidence for this molecule as we identified and characterized by mass spectrometry new estrogenic molecules previously unknown, present in schistosome worm extracts and sera of schistosome infected individuals. The detection method developed by our group by LC–MS analysis specific for estrogenic molecules, revealed that these new estrogens are present in schistosome worm extracts. At the light of these findings, we believe that the species detected on the LC–MS analysis of both the schistosoma extract and the plasma of a *Schistosoma*-infected patient were formed by reactions of estrogen-quinones with DNA. Such reactions might have occurred directly via Michael addition or indirectly through generation of reactive oxygen species (ROS) and led to structures (Scheme 1) compatible with the *m*/*z* values observed (Table 1). To confirm this hypothesis, and given our previous experience in tandem-MS studies to establish fragmentation mechanisms involving compounds relevant in other parasitic diseases (Vale et al., 2008a,b, 2009), we decided to further analyse the serum from a schistosoma-infected individual by LC–MS/MS. Species detected after MS^2 fragmentation of components **1**–**4**, as illustrated in Fig. 7 for component **2** – is the most intense in the schistosoma extract – gave further support to our previous structural assignment for the four components. We chose component **2** because is the most intense with a relative abundance of 100%. Indeed, MS^2 fragmentation of component **2** led to five species that were detected in the negative mode at *m*/*z* values of ca. 595, 621, 677, 695 and 720. These *m*/*z* values are compatible with fragments structures as those shown on Scheme 2, all of which can be easily derived from the parental structure **2**. Identical analyses was done for the remaining three components, **1**, **3** and **4**, whose MS^2 spectra are given in the Supporting Information and also show fragmentation patterns that confirm structures above proposed for those components.

An interpretation of the above findings was built at the light of well-established metabolic pathways for estrogens. It is known from the literature that estradiol is mainly metabolised by (i) oxidation of the hydroxyl function at the C17 position (via steroid dehydrogenase) to yield estrone, and (ii) hydroxylation via cytochrome P450 enzymes, occurring preferentially on C2, C4 and C16 (Bolton et al., 1998; Rathahao et al., 2000). When hydroxylation takes place on the steroid aromatic ring A, catechol-estrogens are produced (Iverson et al., 1996). Similar transformations can easily explain the fact that, on schistosoma samples, each component was associated to two *m*/*z* peaks always differing by 14 a.m.u. In fact, hydroxylation of both C-2 and C-3 on a steroid ring and further oxidation into an estradiol-2,3-quinone (Scheme 3), a phenomenon well described by Iverson and collaborators (1996), would lead to a mass increase of precisely 14 a.m.u.

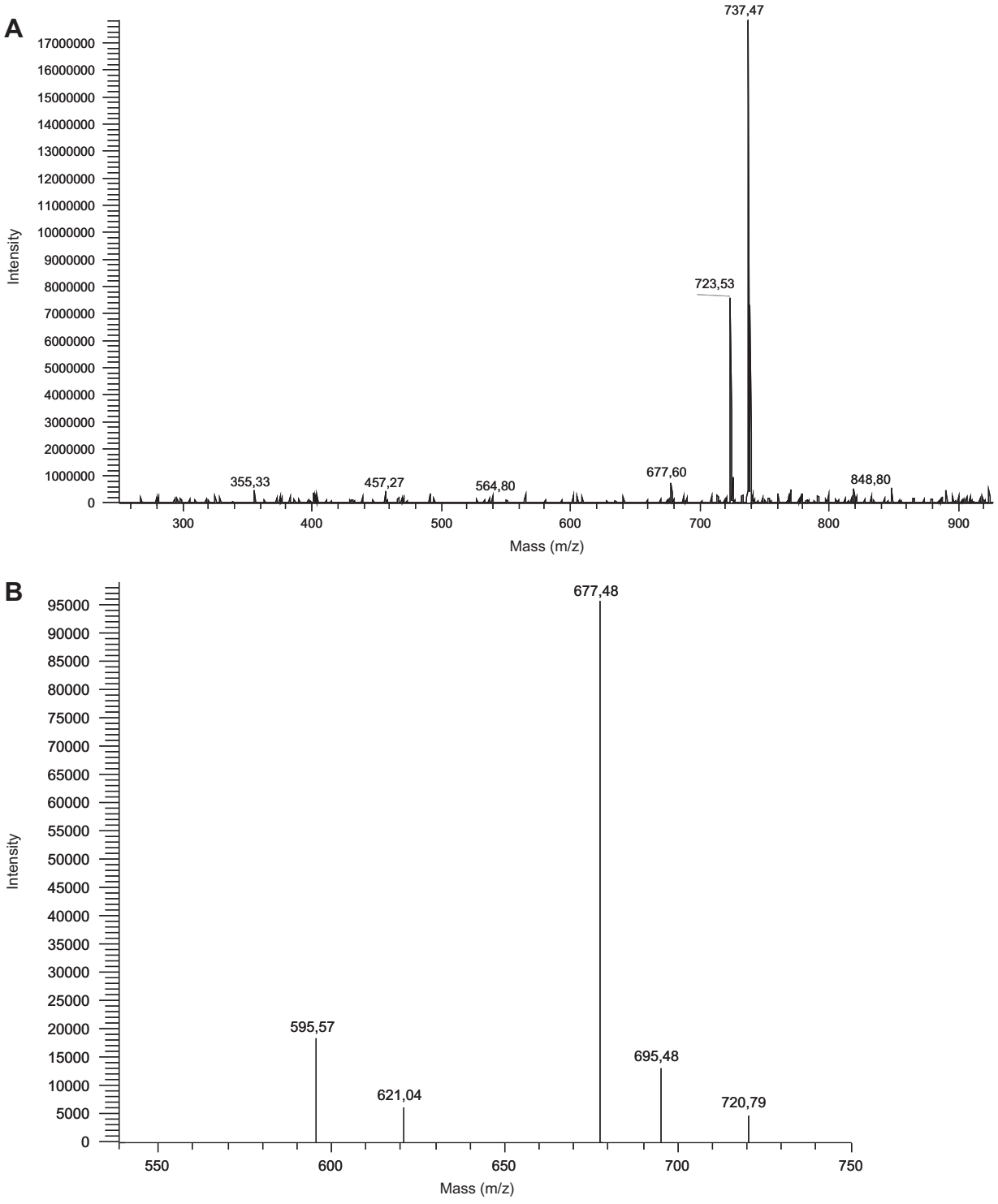
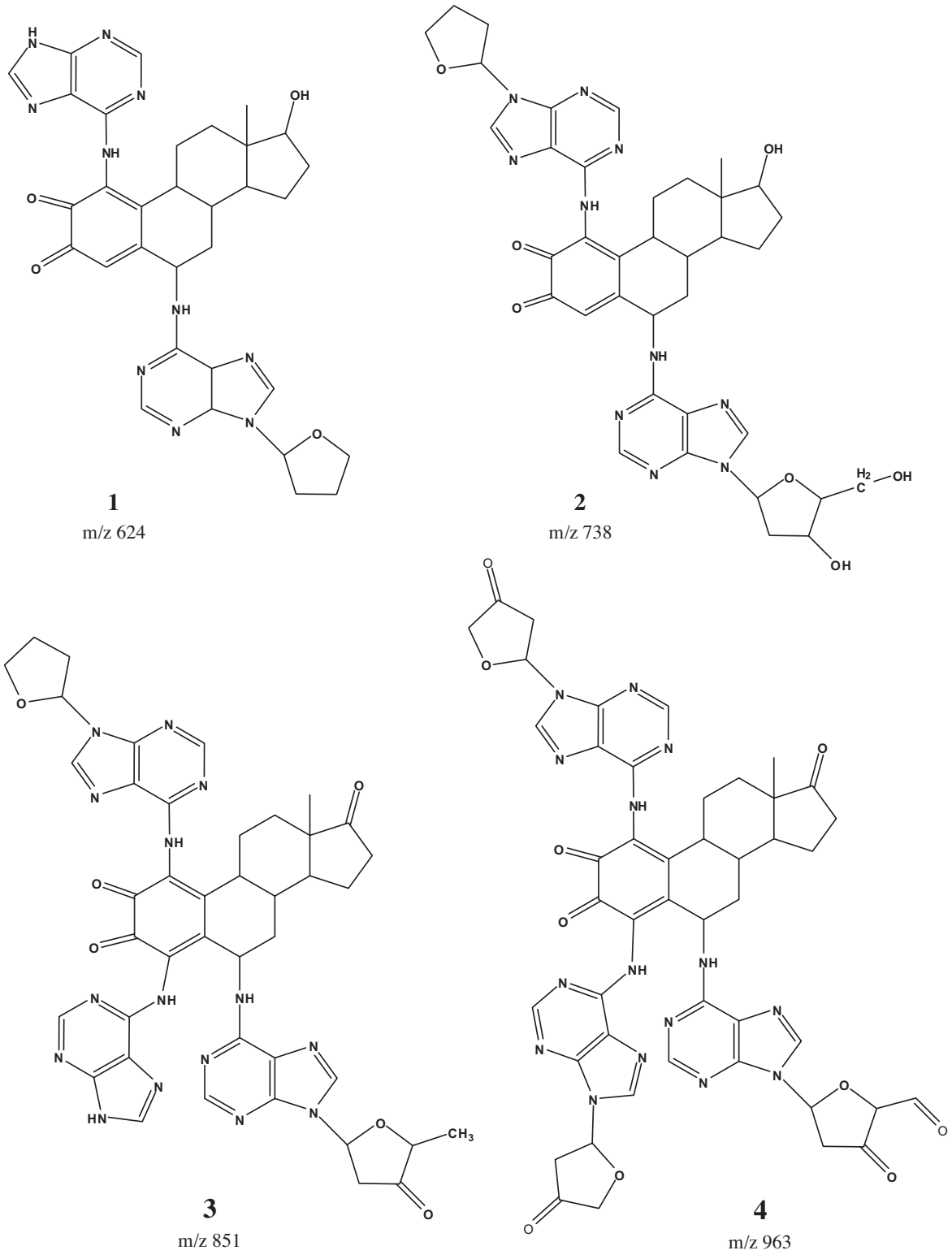


Fig. 7. Mass spectra (MS, (A) and MS^2, (B)) for component **2**, detected in the LC–MS/MS analysis of *Schistosoma*-infected human serum. MS and MS^2 spectra for components **1**, **3** and **4** are given on Supporting Information.

Furthermore, the proposed molecular structure of these new estrogenic molecules explains the cellular effects of Sh demonstrated in this paper. These molecules show a putative binding affinity with ER and they probably are able to antagonize estradiol activity and down-regulate ER.

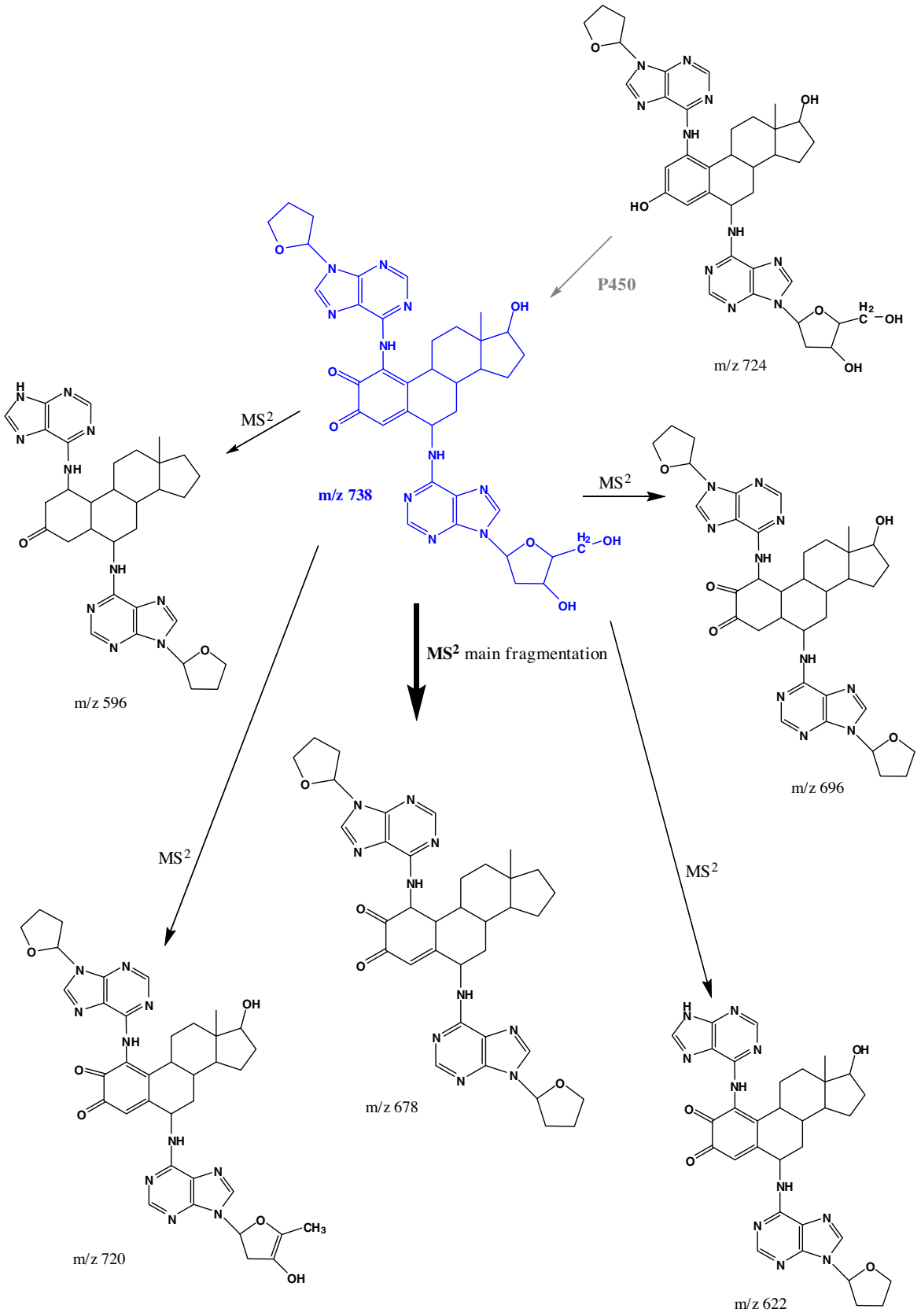
Finally, to our knowledge there are no studies in the literature reporting the presence of genes involved in estrogen pathways in *S. haematobium*. As the genome sequences of the closely related African schistosome *Schistosoma mansoni* (Berriman et al., 2009) and the more distantly related Asian species *Schistosoma japonicum* (The Schistosoma japonicum Genome Sequencing and Functional Analysis Consortium, 2009) are now available we can expect that schistosomes will express all the genes necessary to synthesize estrogenic molecules. In this regard, it is noteworthy that the genomic information suggests the presence of an integral hypothalamic–pituitary–thyroid in *S. japonicum*. It was also confirmed that *S. japonicum* has receptors for steroid hormones such as progestin, progesterone and estrogen. In addition it possesses intricate



Scheme 1. Components **1**, **2**, **3** and **4**.

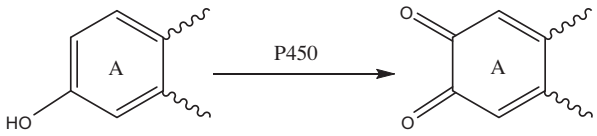
pathways for processing steroid hormones to form other sex hormones. For example, there are putative enzymes present that could convert the female hormones progesterone and pregnenolone to estriol, oestrone, androsterone and testosterone. Hence, schistosomes might use these pathways during their parasitic existence (The Schistosoma japonicum Genome Sequencing and Functional Analysis Consortium, 2009).

The observations described in this study assist in defining the biochemical basis of the selective interactions between Sh and the ER, and they provide insights into the molecular mechanisms by which Sh acts to modulate the activity of this steroid hormone receptor, possibly through the new estrogenic molecules identified in the crude parasite extracts. Additional studies addressing the regulation of expression of these new estrogenic molecules of



Scheme 2. Proposed MS^2 fragmentation pathway for component **2**.

S. haematobium and their antiestrogenic activity will be important in further understanding how this parasite infection influences ER transcription, as well as the benefit this ER repression may provide to the parasite.



Scheme 3. Hydroxylation of both C-2 and C-3 on a steroid ring and further oxidation into an estradiol-2,3-quinone.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.exppara.2010.06.012.

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